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Comparison of biomarkers in workers exposed to 2,4,6-trinitrotoluene

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Abstract

2,4,6-Trinitrotoluene (TNT) is an important occupational and environmental pollutant. In TNT-exposed humans, notable toxic manifestations have included aplastic anaemia, toxic hepatitis, cataracts, hepatomegaly, and liver cancer. Therefore, methods were developed to biomonitor workers exposed to TNT. The workers were employed in a typical ammunition factory in China. The external dose (air levels and skin exposure), the internal dose (urinary metabolites), the biologically effective dose (haemoglobin adducts, urinary mutagenicity), biological effects (chromosomal aberrations and health effects), and individual susceptibility (genotypes of xenobiotic-metabolizing enzymes) were determined. Haemoglobin-adducts of TNT, 4-amino-2,6-dinitrotoluene (4ADNT) and 2-amino-4,6-dinitrotoluene (2ADNT), and the urinary metabolites of TNT, 4ADNT and 2ADNT, were found in all workers and in some controls. The levels of the haemoglobin-adducts or the urinary metabolites correlated weakly with the skin or air levels of TNT. The urinary mutagenicity determined in a subset of workers correlated strongly with the levels of 4ADNT and 2ADNT in urine. The haemoglobin-adducts correlated moderately with the urinary metabolites and with the urinary mutagenicity. The genotypes of glutathione *S*-transferases (*GSTM1, GSTT1, GSTP1*) and *N*-acetyltransferases (*NAT1, NAT2*) were determined. In general, the genotypes did not significantly influence the haemoglobin-adduct levels and the urine metabolite levels. However, TNT-exposed workers who carried the *NAT1* rapid acetylator genotype showed an increase in urinary mutagenicity and chromosomal aberrations as compared with slow acetylators. The haemoglobin adduct 4ADNT was significantly associated with a risk of hepatomegaly, splenomegaly and cataract; urine metabolites and genotypes were not associated with health effects. These results indicate that a set of well-selected biomarkers may be more informative regarding exposure and effect than routinely performed chemical measurements of pollutants in the air or on the skin.

Keywords: *Chromosomal aberrations, GSTM1, GSTP1, GSTT1, haemoglobin-adducts, NAT1, NAT2, TNT, urinary metabolites, urinary mutagenicity*

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Introduction

2,4,6-Trinitrotoluene (TNT) is an important environmental and occupational pollutant (Hathaway 1985, Yinon 1990, IRIS 1991, Rosenblatt et al. 1991, ATDSR 1995). In TNT- exposed humans, notable toxic manifestations have included aplastic anaemia, toxic hepatitis and cataracts (Hathaway 1985, Rosenblatt et al. 1991, ATSDR 1995). In China, chronic occupational exposure to TNT has caused hepatomegaly and cataracts (Qu 1984, Liu et al. 1995). A recent retrospective study on male workers exposed to TNT for more than 1 year from eight Chinese military factories from 1970 to 1995 demonstrated an elevated relative risk for malignant tumours, especially liver cancer (Yan et al. 2002).

In vitro studies with rat liver microsomes showed that TNT is rapidly reduced to yield 4-hydroxylamino-2,6-dinitrotoluene (OH-4ADNT), 4-amino-2,6-dinitrotoluene (4ADNT) and 2-amino-4,6-dinitrotoluene (2ADNT) as intermediates that are further metabolized to form 2,4-diamino-6-nitrotoluene (24DANT) and 2,6-diamino-4-nitrotoluene (26DANT) (Leung et al. 1995). The *N*-hydroxyarylamine (OH-4ADNT) of 4ADNT was stable enough so that the reduction of TNT to 4ADNT appeared to be a two-step pathway (Yinon 1990, Leung et al. 1995). In contrast, the 2ADNT intermediate, OH-2ADNT, could not be isolated.

Aminonitrotoluenes can be *N*-oxidized by cytochromes P450 1A2 (CYP1A2) and 3A4 (CYP3A4) to yield *N*-hydroxyarylamines (Kim & Guengerich 2005). The oxidation of the methyl group in animals appears to be a minor pathway because such products have been reported only once in the literature (reviewed in Yinon 1990). The hydroxy-group of the *N*-hydroxyarylamines and the amino-group of 4ADNT, 2ADNT, 24DANT and 26DANT may undergo conjugation reactions with sulfuryl, glucuronide or acetyl moieties. These metabolic steps are catalysed in part by enzymes present in polymorphic forms in humans, e.g. the sulfotransferases 1Al (SULT1A1) and 1A2 (SULT1A2), and the *N*-acetyltransferases 1 (NAT1) and 2 (NAT2) (Beland & Kadlubar 1990, Delclos & Kadlubar 1997, Glatt 2000). Secondary products of *N*-hydroxyarylamines are responsible for the genotoxic and cytotoxic effects of these compounds.

Detoxification products are formed after *N*-acetylation catalyzed by NAT1 or NAT2 and by reaction with glutathione, with or without glutathione *S*-transferase (GST) catalysis (Coles & Kadlubar 2003). The specific *GST* isoforms involved in the detoxification process are presently unknown. It has been shown *in vitro* that glutathione decreases the amount of reactive TNT intermediates. In cotton rats exposed to TNT, the activity of CYPs and hepatic GSTs was significantly elevated (Reddy et al. 2000). In rat liver cytosol, *N*-acetylation was demonstrated with NAT1 and NAT2 (Li et al. 1998). *In vivo* such reaction products have not been isolated. In mammalian systems the principal metabolites of TNT are 2ADNT and 4ADNT; smaller amounts of other metabolites have also been found (Yinon 1990).

In humans, mainly 2ADNT and 4ADNT were found in urine after TNT exposure (Woollen et al. 1986, Ahlborg et al. 1988, Yinon 1990, Bader et al. 1998). Urinary metabolites usually indicate only recent exposures up to 48-h post-exposure (Van Welie et al. 1992). Haemoglobin (Hb)-adducts are an indicator of exposure over the last 4 months, if the adduct is stable and if the lifetime of the erythrocytes is not affected. The mechanism of adduct formation between aromatic amines or nitroarenes with Hb involves the reaction of the metabolite, nitrosoarene, with cysteine residues to form a sulfinic acid amide (Neumann 1984, Green et al. 1984, Ringe et al.

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1988, Kazanis & McClelland 1992, Sabbioni & Jones 2002). Sulfinic acid amide adducts are readily hydrolysed under mild conditions, yielding the parent amine.

Hb adducts are dosimeters for the internal dose and possibly for the target dose leading to toxic effects. Covalent Hb, plasma protein and protein adducts in various tissues were found in rats dosed with radio labelled TNT (Liu et al. 1992). Therefore, Hb-adducts of TNT might be a biomarker of toxic effects. The presence of two Hb-adducts (Sabbioni et al. 1996) indicates that OH-4ADNT and OH-2ADNT are biologically available. These intermediates can be formed by reduction of TNT in the gut, in the liver, and in the erythrocytes (Liu et al. 1992, Leung et al. 1995). Hb-adducts have been found in workers (Liu et al. 1995, Sabbioni et al. 1996).

According to the paradigm of biomonitoring studies, the following parameters were compared in the present study: (1) external exposure (air and skin monitoring), (2) internal exposure (urine metabolites), (3) biologically effective dose (Hb-adducts and urinary mutagenicity), (4) individual susceptibility (genotypes of the nitrotoluene-metabolizing enzymes *GSTM1, GSTP1, GSTT1, NAT1* and *NAT2*), and (5) biological effects (chromosomal aberrations, clinical blood and urine parameters, splenomegaly, cataracts, and hepatomegaly). The results with the various biomarkers were compared with traditional measures of exposure involving the levels of TNT in the air and on the skin.

Materials and methods

Workers

The workers for this study were recruited from the Tuoli Chemical Factory, which is located in a suburb of Beijing, China. The workers ($n = 78$) were mostly involved in the production of satchel charges for mining. The main processes involved were mixing ($n = 35$), loading ($n = 26$), grinding ($n = 10$), and packing ($n = 6$). The time of exposure was 8 h per day and 5 days per week. The factory controls ($n = 25$) were employed in the same factory but were no longer working directly in jobs that would expose them to nitrotoluenes. Of the 25 controls, eight had been exposed for 8–20 years to TNT up to two or more years ago. All exposed workers, except two, were males. The control group consisted of nine females and 16 males. The mean ages of the controls and exposed group were 38.4 ± 9.4 and 39.6 ± 8.1, respectively. The mean work-years of the controls and exposed group were 15.1 ± 9.7 and 9.8 ± 7.0, respectively. Among the control and exposed workers, 64 and 84% were smokers, respectively.

For the cytogenetic studies, another control group of 26 occupationally unexposed subjects (24 men and two women) was recruited from the Institute of Occupational Medicine, Beijing (laboratory controls). The additional control group was needed to exclude possible clastogenic effects of past exposure of the factory controls to nitrotoluenes and was sampled concurrently with the TNT-exposed workers. The mean age of the laboratory controls was 34.7 ± 9.1, and 62% of them were smokers.

The study was performed in accordance with the principles embodied in the Declaration of Helsinki (http://www.wma.net/e/policy/b3.htm). Informed consent was obtained from each worker. The collection of blood and urine, medical examination, air sampling, and completion of questionnaires were all performed in the same week. Participants were interviewed with a questionnaire about their general health status, exposure history, smoking habits, previous medical record, and present symptoms. The medical department of the Chinese Academy of Preventive Medicine performed

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the following examinations: (1) physical examination, including the cardiovascular system, (2) routine blood tests, including liver function tests measuring glutamic pyruvic transaminase, alkaline phosphatase, total protein, albumin, total bilirubin, (3) electrocardiogram (ECG): ECG1 = sinus tachycardia, sinus bradycardia, ECG2 = arrhythmia, ECG3 = abnormal conduction, (4) ultrasonic type examination for liver and spleen, (5) serological assays of hepatitis B, and (6) ophthamaological examinations. Cataract, splenomegaly, hepatomegaly, ECG1, ECG2, and ECG3 data were obtained from 68, 75, 72, 65, 78 and 65 exposed workers, respectively; and from 25 factory controls except for cataract ($n = 23$). All the other tests were performed on all workers and controls.

External exposure

Skin and air exposure was determined in 32 and 31 workers, respectively. For the determination of the air levels of TNT, we used method 44 of OSHA (http://www.osha-slc.gov/dts/sltc/methods/organic/org044/org044.html). Tenax GC tubes were used to collect TNT air samples. The air was sampled by the aid of a pump on glass fibre filters connected to ATD-Tenax-TAX tubes to ensure that both dust and vapours were trapped. The velocity of the pumps was 100–200 ml min^{-1}, enabling us to sample 30–60 dm^3 air for 4–5 h a day. The samples were collected from four different worksites, including grinding (six workers), loading (14 workers), mixing (nine workers), and packing (one worker). Only the grinders were exposed to pure TNT (100%). The other workers dealt with a blend containing wooden powder, 11% TNT, and a few per cent ammonium nitrates. The Tenax tubes were thermo-desorbed and analysed by gas chromatography (GC) equipped with an electron capture detector (ECD); the filters were desorbed in toluene and analysed by GC-ECD. Skin exposure was determined as described by Liu et al. (1995), and TNT was analysed by using GC-ECD to measure skin contamination. Fixed areas of the body surface area (forehead, neck, upper arms, hands, back abdomen, thighs, shanks and feet) were smeared with cotton swabs soaked with ethanol. All the results were calculated according to the smear area (approximately 336.4 cm^2 for each person) and adjusted with participant's body height and body weight (Liu et al. 1995).

Urinary metabolites

Urinary metabolites were determined and have been published in Sabbioni et al. (2005). Urinary metabolites were analysed with and without β-glucuronidase treatment from the urine of 71 workers and eight controls.

Haemoglobin-adducts

Hb-adducts of TNT were analysed and described in Sabbioni et al. (2005). Samples of 78 workers and 25 controls were analysed.

Urinary mutagenicity

Organic extracts of urine samples were prepared from unhydrolysed, enzymatically hydrolysed, or acid-hydrolysed urine as described previously (Kato et al. 2004). Briefly, the enzymatic hydrolysis involved incubation of urines at 37°C for 3 h with

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β-glucuronidase (Sigma, St Louis, MO, USA) and arylsulfatase type H-2 from *Helix pomatia,* EC 3.1.6.1 (Sigma). For the acid hydrolysis, urines were incubated at 70°C for 6 h in 6 M HCl and then neutralized by the addition of 6 M NaOH and $NaHCO_3$. The organics from the unhydrolysed or hydrolysed urines were then extracted by passing the samples through C18 resin and eluting the organics with methanol. The organics were solvent exchanged into dimethyl sulfoxide at a concentration that was 150-fold more concentrated than in urine. Urinary mutagenicity was assessed with the *Salmonella* plate-incorporation assay (Maron & Ames 1983). The frameshift strain YG1041 (*hisD3052*, *rfa*, *ΔuvrB*, pKM101) was used, which also has elevated acetyltransferase and nitroreductase activities due to plasmid-mediated gene amplification (Hagiwara et al. 1993). Extracts were evaluated at 0, 0.15, 0.75, 1.5, 3.0, and 7.5 ml-eq./plate in the absence of S9 mix. Mutagenic potencies (revertants/ml-eq.) were calculated from the linear portion of the dose–response curves. Urinary mutagenicity was determined on 11 workers and six controls.

Genotype analyses

Genomic DNA from 78 exposed workers and 25 controls was extracted from lymphocytes by standard techniques (Hirvonen et al. 1996, Saarikoski el al.1998). The *GSTM1* and *GSTT1* genotypes were determined from the genomic DNA by a multiplex PCR analysis. In this analysis, *GSTM1*- and *GSTT1*-specific primer pairs were used together with a third primer pair for β-globin in a multiplex PCR analysis. The absence of the *GSTM1*- or *GSTT1*-specific PCR-product indicated the corresponding null genotype, whereas a β-globin-specific fragment confirmed proper functioning of the reaction (Hirvonen et al. 1996, Saarikoski et al. 1998). Similarly, in the *GSTP1*-genotyping, the variant alleles containing a base substitution at nucleotide 313 (*GSTP1*B* and *GSTP1*C*) resulting in Ile105Val amino acid change were differentiated from the wild-type allele (*GSTP1*A*) by *SnaBI* restriction enzyme digestion subsequent to a PCR amplification (Saarikoski et al. 1998). Because this method did not differentiate between *GSTP1*B* and *GSTP1*C* alleles, the Val 105 alleles were designated as *GSTP1* Val.

The *NAT1* alleles (**3*, **4*, **10*, **11*) and *NAT2* alleles (*4*, **5*, **6*, **7*) were determined as previously described (Bell et al. 1993, 1995). To ensure laboratory quality control, two independent readers interpreted the results. Any sample with ambiguous results was retested, and a random selection of 10% of all of the samples was repeated. No discrepancies were discovered upon replicate testing.

Genotype classification

The *GSTM1* and *GSTT1* genotypes were classified as null and positive genotypes, while the *GSTP1* genotypes were dichotomized into homozygous Ile/Ile genotypes and Val-allele carrying genotypes (Ile/Val and Val/Val). For *NAT1,* the *NAT1*10* and *NAT1*11* alleles were classified as rapid alleles. The wild-type-like alleles *NAT1*3* and *NAT1*4* were considered comparable and classified as normal acetylation alleles. Two groups were formed: rapid acetylators (individuals with one or two rapid alleles) and slow acetylators (individuals with two normal alleles). For *NAT2,* the *NAT2*4* allele was considered as the rapid allele, and the *NAT2*5, NAT2*6* and the *NAT2*7* as the slow alleles. The genotyping method used (Bell et al. 1993) did not differentiate

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between the *NAT2*5A* and **5B*, *NAT2*6A* and **6B*, and *NAT2*7A* and **7B* alleles, respectively. *NAT2* genotypes were divided in two groups: the homozygous slow acetylators (individuals with two slow alleles) and the rapid acetylators (individuals with one or two fast alleles).

Chromosome aberration assay

Samples of heparinized peripheral blood (5 ml) were collected from the exposed workers and controls for the chromosome aberration assay. Two lymphocyte cultures per sample were established in 20-ml vials within 24 h after the sampling. Each culture contained 0.3 ml of whole blood and 6 ml of culture medium consisting of 97% (v/v) of RPMI 1640 medium without serum (GIBCO, Glasgow, UK), 1% of phytohaemagglutinin (Murex, Dartford, UK), 1% of 200-mM L-glutamine solution (GIBCO), 1% of penicillin-streptomycin solution (100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin; GIBCO) (Sabbioni et al. 2006). The cultures were incubated at 37°C for 44 h. Colcemid solution (70 μl, 10 μg ml^{-1}) was added to the vials 2.5 h before the harvest to arrest mitotic cells in metaphase. The cells were harvested by centrifugation, treated with a hypotonic solution (0.075 M KCl) at 37° C for 8 min and fixed three times in methanol-glacial acetic acid (3:1). The duplicate cell suspensions of each sample were combined after the second fixation. From each tube, six to eight microscope slides were prepared by dropping a few drops of the cell suspension on wet glass slides. The slides were air dried, stained in Giemsa (4%, in Sorensen buffer, pH 7.0, 5 min), and coded for the analysis.

Up to 100 metaphases from each individual were analysed for chromosomal aberrations using Cytogenetics Image Analysis System CS2 metaphase finder (Cytoscan; Image Recognition Systems, Warrington, UK). One laboratory technician performed all the scoring. The analysis of chromosomal aberrations was done according to ISCN (1985). When the analyses were completed, the code was broken.

Statistical analysis

Statistical analyses were performed with the SPSS program (version 10.0). The results of the questionnaire and of the medical examination were not known to the scientists performing the analyses of the biomarkers. All results were disclosed at the end of the analyses. For a comparison of one dichotomous dependent variable with a continuous independent variable, the Mann–Whitney *U*-test was used. For the comparison of two sets of dichotomous variables, contingency tables were used.

Results and discussion

External dose: air and skin measurements

Standard procedures were followed to determine the air and skin concentration of TNT. The mean 8-h time-weighted average (TWA) exposure in μg m^{-3} is listed in Table I. The occupational exposure limit set by the US National Institute of Occupational Safety and Health (NIOSH) is 1.5 mg m^{-3} for TNT. This limit was exceeded in 35% of the workers. The skin exposure was comparable with the levels determined in an earlier study (Liu et al. 1995). The levels of TNT in air and skin correlated (Table II).

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Internal dose: urinary metabolites

The methods and results of the urinary metabolites resulting from the exposure to TNT have been described in detail by Sabbioni et al. (2005). Urine samples were analysed with and without β-glucuronidase treatment. Among the exposed workers, 100 and 97% urinary samples were positive for 4ADNT and 2ADNT (Table I), respectively, in extracts of raw urine or in extracts of enzymatically hydrolysed urine. Among the control workers, 50% of the urine samples were positive for 4ADNT and 2ADNT after both work-up procedures. The levels of 4ADNT and 2ADNT in urine samples without enzyme treatment were much lower than after enzyme treatment; the mean levels of 4ADNT and 2ADNT were 3.6 and 2.4 times lower in raw urine than in enzyme-treated urine. Levels of urinary metabolites in control samples were significantly lower than in the exposed workers.

Biologically effective dose: haemoglobin-adducts

The Hb-adducts of 4ADNT and 2ADNT were found in 100 and 81%, respectively, of the exposed workers (Sabbioni et al. 2005). 4ADNT and 2ADNT (Table I), respectively, were found in 16 and 8% of the factory controls. The Hb-adduct levels in the exposed workers were significantly higher than in the factory controls ($p < 0.01$; Mann–Whitney *U*-test). The mean levels of 4ADNT were about 28 times higher than the levels of 2ADNT. Therefore, 4ADNT, but not 2ADNT, was found in most workers. The levels of 4ADNT and 2ADNT were correlated ($r = 0.81$, Table II). The mean levels of 4ADNT found in the present factory were similar to the mean levels found in a previously studied ammunition factory (110 ng 4ADNT g^{-1}Hb) (Sabbioni et al. 1996).

Biologically effective dose: Urinary mutagenicity

Urinary mutagenicity was determined in a subset of exposed and control workers. The mean mutagenic potencies (rev/ml-eq.) of the unhydrolysed, enzymatically hydrolysed, and acid-hydrolysed urines were, respectively, 3.9 ± 2.5, 8.8 ± 7.6 and 2.8 ± 3.2 in the controls and 198.8 ± 375.8, 486.4 ± 535.9 and 53.7 ± 76.5 in the exposed workers. The median levels were significantly higher in the exposed workers (Mann–Whitney *U*-test, $p = 0.001$). The mutagenicity of the unhydrolysed urine correlated (Spearman rank correlation) with that of the enzymatically hydrolysed urine ($r = 0.94$, $p < 0.001$) and acid-hydrolysed urine ($r = 0.82$, $p < 0.001$). The mutagenicity of the

Table I. Comparison of the mean (±SD) air levels, urine levels and haemoglobin (Hb)-adduct levels found in exposed workers.

Compound	Air: TWA[a] (mg m^{-3})	Skin (mg)	Urine compound	Urine enzyme-treated (μg l^{-1})	Urine raw (μg l^{-1})	Hb-adducts compound	Hb-adducts (ng g^{-1} Hb)
TNT	1.28 ± 1.06	110 ± 222	4ADNT	1098 ± 870	307 ± 310	4ADNT	90.7 ± 136
			2ADNT	303 ± 258	124 ± 890	2ADNT	3.20 ± 3.67

[a]TWA = 8-h time weighted average.

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enzymatically and acid-hydrolysed urines correlated with each other ($r = 0.92$, $p < 0.001$).

Correlation of skin levels, air levels, urine levels, and haemoglobin-adducts

Spearman rank correlations between the external, internal, and biologically effective exposures are summarised in Table II. The different markers were not available for all workers. In the same category, biomarkers correlated well among each other, e.g. Hb-adducts of 4ADNT versus Hb-adducts of 2ADNT. The skin levels correlated weakly with the levels of a urinary metabolite released after enzyme treatment and moderately with the Hb-adducts. The air levels correlated only with one of the urinary metabolites released after enzyme treatment. The urine levels of 4ADNT in enzyme-treated urine correlated with the levels of 4ADNT in raw urine (Table II). The levels of 4ADNT and 2ADNT correlated in enzyme-treated as well as raw urine. The levels of urinary metabolites correlated weakly with the levels of Hb-adducts.

Correlation of urinary mutagenicity with urinary metabolites and haemoglobin-adducts

The correlations between urinary mutagenicity and other biomarkers for exposed workers are summarized in Table II. The correlations between mutagenicity and the other biomarkers improved if the samples of the factory controls were included (data not shown in Table II). For all exposed workers and controls, the levels of urinary metabolites, U-gl-4ADNT, determined in enzymatically hydrolysed urine, correlated the best with mutagenicity ($r = 0.89–0.96$, $p < 0.01$; Spearman-rank test). The best correlation was found with mutagenicity and U-gl-4ADNT where both values were obtained from enzymatically treated urine. Unhydrolysed 4ADNT also correlated with urinary mutagenicity ($r = 0.83–0.92$, $p < 0.01$). The correlations of the urinary mutagenicity with 2ADNT were in general lower: $r = 0.85–0.92$ for U-gl-2ADNT and 0.79–0.90 for 2ADNT ($p < 0.01$). Excellent correlations ($p < 0.01$) were also found for mutagenicity with the Hb-adduct 4ADNT ($r = 0.79–0.89$) and 2ADNT (0.72–0.78). Thus, the exposure to TNT appeared to be important for the observed urinary mutagenicity. Hb-adduct levels correlated with the urinary mutagenicity. Therefore, Hb-adducts were a good marker of exposure for predicting the mutagenic activity present in the urine.

Individual susceptibility: genotypes of exposed and control subjects

The genotypes found in the Chinese workers are presented in Table III. The *GSTT1* and *NAT2* genotypes were distributed equally among the controls and exposed workers, whereas a deviant distribution was observed for *GSTM1*, *GSTP1* and *NAT1*. The difference was statistically significant for *GSTM1* and for *GSTP1* (Fisher's exact test, $p < 0.01$). In the exposed group, workers carrying the *GSTM1* gene and homozygous for the *GSTP1* wild-type allele were more prevalent than in the control group. This has also been noted for *GSTM1* in workers in another nitrotoluene factory (Sabbioni et al. 2006). Thus, it is possible that adverse effects deter *GSTM1*-deficient subjects from working with nitrotoluenes in general. For *GSTP1* it appeared that in the exposed workers the *GSTP1* Val-allele containing genotypes were underrepresented. This might be due to the fact that *GSTP1* Ile/Ile genotype poses a protective effect on the formation of reactive intermediates, as has been shown for

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Table II. Spearman rank correlation between air levels, urine levels, and haemoglobin (Hb)-adduct levels in workers exposed to TNT.

	Air	Skin	U 4ADNT	U 2ADNT	U-gl 4ADNT	U-gl 2ADNT	Hb 4ADNT	Hb 2ADNT	U mut-enz	U mut-raw
Skin	0.69[a]									
U-4ADNT	−0.02	0.01								
U-2ADNT	−0.09	0.04	0.85[a]							
U-gl-4ADNT	0.41[b]	0.35	0.76[a]	0.65[a]						
U-gl-2ADNT	0.35	0.39[b]	0.69[a]	0.73[a]	0.85[a]					
Hb-4ADNT	0.28	0.54[a]	0.31[a]	0.25[b]	0.44[a]	0.48[a]				
Hb-2ADNT	0.18	0.35	0.37[a]	0.35[a]	0.46[a]	0.54[a]	0.86[a]			
U-mut-enz	0.80	0.03	0.71[b]	0.64[b]	0.89[a]	0.75[b]	0.49	0.58		
U-mut-raw	0.70	0.14	0.55	0.52	0.76[b]	0.52	0.68[b]	0.50	0.77[a]	
U-mut-acid	−0.70	−0.60	0.82[a]	0.87[a]	0.72[b]	0.78[a]	0.38	0.54	0.75[a]	0.40

U-mut-acid and U-mut-enz are the mutagenicity found in acid and enzyme-treated urine, respectively. Statistically significant correlations are marked thus: (a) $p < 0.01$, (b) $p < 0.05$.

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Table III. Genotype frequencies in TNT-exposed workers and factory controls.

Group	Number of subjects	*GSTM1* null	*GSTP1* Ile/Val+Val/Val	*GSTT1* null	*NAT1*[b] slow	*NAT2* slow
Exposed workers	78	50%[a]	4%[a]	36%	31%	28%
Factory controls	25	80%[a]	32%[a]	36%	16%	24%

[a]Statistically significantly different distribution (Fisher's exact test, $p < 0.01$).
[b]*NAT1* genotype could not be determined in one exposed worker.

TNT-Hb-adduct formation in workers exposed to a mixture of mononitrotoluenes, dinitrotoluenes and TNT (Sabbioni et al. 2006). The mean Hb-adduct levels of TNT in that study were 26 times lower than in the present population. In the population with lower TNT exposure, the *GSTP1* genotype distribution was similar in controls and exposed workers (Sabbioni et al. 2006). Therefore, workers carrying the *GSTP1* Val-allele may have been transferred to other factory departments because they were more susceptible to health effects resulting from TNT exposure.

Individual susceptibility: genotype, haemoglobin-adducts, urinary metabolites and urinary mutagenicity

Urinary mutagenicity was significantly higher ($p < 0.05$) in workers with *NAT1* rapid acetylator genotype (515 = median, $n = 7$) than in those with the slow acetylator genotype (124, $n = 4$); no other genotypes showed a significant influence on urinary mutagenicity.

In a first analysis, the genotypes were correlated with the Hb-adduct (4ADNT) and the urinary metabolite level from enzymatically treated urine (U-gl-4ADNT) without taking into account personal exposures (data not shown). The median Hb-adduct levels were increased in exposed workers with the *GSTM1* positive genotype and *NAT2* rapid acetylator genotype, but decreased in workers with the *GSTP1* Val-allele, the *GSTT1* positive genotype, and the *NAT1* rapid acetylator genotype. The same trend was seen also for the median urinary metabolite levels, except for *GSTT1* and *NAT2* genotypes. However, the differences between the genotypes in biomarker levels were not statistically significant in any instance.

In a further analysis, combined effects of genotypes on the Hb-adduct levels were examined; none of the combinations yielded significant differences in Hb-adduct levels (Table IV). The combinations of all genotypes (except for the *GSTT1* null) with *GSTM1* showed an increase of the adduct levels in workers with the *GSTM1* present genotype. All genotypes (except for the *GSTM1* null) yielded a decrease of the adduct level in combination with the *NAT1* rapid acetylator genotype. A similar trend was seen for the urinary metabolites.

In the next step, the Hb-adduct levels were correlated to the air levels by taking into account the genotypes. We expected to see an improvement in the correlations in comparison with the analysis where the genotypes were not taken into consideration. This was the case only for workers with the *GSTM1* null genotype, in whom the correlation increased to $r = 0.65$. For the other genotypes the correlation coefficient decreased or remained the same. For the urinary metabolite U-gl-4ADNT

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Table IV. Combination of genotypes and effect on the median adduct (Hb-4ADNT) and the median urinary metabolite (U-gl-4ADNT) levels in TNT-exposed workers.

	GSTM1 null versus positive	*GSTT1* null versus positive	*GSTP1* Ile/Ile versus Ile/Val, Val/Val	*NAT1* slow versus rapid	*NAT2* slow versus rapid
GSTM1 null		**63**/47[a]	–[c]	47/**54**	**57**/50
		80/**102**[b]		**100**/63	**95**/55
GSTM1 positive		59/**69**	**69**/42	**69**/68	**69**/68
		70/**110**	**99**/82	**114**/87	**111**/94
GSTT1 null	**63**/59		–[c]	**69**/53	56/**64**
	80/70			**81**/63	**88**/62
GSTT1 positive	47/**69**		**58**/42	53/**60**	**69**/53
	102/110		**107**/82	**112**/99	**108**/102
GSTP1 Ile/Ile	52/**69**	**63**/58		**63**/56	58/**62**
	81/**99**	**76**/107		**108**/76	**105**/86
NAT1 slow	47/**69**	**69**/53	–[c]		62.8/**63.3**
	100/**114**	81/**112**			100/**115**
NAT1 rapid	54/**68**	53/**60**	**56**/42		**56**/54
	63/**87**	63/**99**	**76**/82		**119**/70
NAT2 slow	57/**69**	56/**69**	–[c]	**63**/56	
	95/**111**	88/**108**		100/**119**	
NAT2 rapid	50/**68**	**64**/53	**62**/24	**63**/54	
	55/**94**	**86**/75	**86**/75	**115**/54	

[a]Median Hb-4ADNT adduct level (ng g^{-1}Hb).
[b]Median U-gl-4ADNT metabolite level (ng/100 μl).
[c]All *GSTP1* Ile/Ile.

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determined in enzymatically treated urine, the influence of the genotype was more pronounced. In workers with *GSTM1* null or the *GSTT1* null genotype, the correlation increased to $r=0.69$. A smaller increase was noted in workers with the *NAT1* and the *NAT2* genotype with $r=0.45$ and 0.52, respectively (Table II).

Biological effects: clinical parameters

The clinical blood parameters of the exposed male workers were compared with the genotypes and with the Hb-adduct levels using the Mann–Whitney *U*-test. The Hb-adduct levels were categorized in two groups. Hb concentration was significantly higher in workers with the *NAT1*-rapid genotype, and the levels of white blood cells were significantly higher in workers with the *NAT2* rapid acetylator genotype. Other comparisons with the genotypes did not yield statistically significant differences. Exposed workers with Hb-adduct levels above the median showed a significant decrease ($p<0.05$) of Hb-concentration, the number of red blood cells, hematocrit, the number of white blood cells, sgpt, and total protein (Sabbioni et al. 2005).

Biological effects: health effects and biomarkers

In the present study, cataracts, hepatomegaly, splenomegaly, hepatitis B, and the cardiovascular effect ECGl were more prevalent in the exposed workers than in the factory controls (60 versus 26%, 22 versus l2%, 13 versus 0%, 29 versus 20%, and 22 versus l2%, respectively). Cardiovascular alterations as measured by ECG2 and ECG3 were more prevalent in the control workers than in the exposed workers. The differences between the TNT-exposed workers and the factory controls were statistically significant (Fisher's exact test, $p<0.05$) for cataract, splenomegaly, and ECG1. The health effects were compared with the different biomarkers, and the odds ratio (OR) for workers carrying a specific genotype were calculated with contingency tables. All results were statistically non-significant. Workers carrying the *GSTT1* null genotype had an OR of 2.4 (confidence interval, CI = 0.85–6.8) to develop cataract, and an OR of 6.4 (CI = 0.76–53.4) for the cardiovascular effect ECG3 (abnormal conduction).

The influence of Hb-adduct levels on the OR of developing a disease was estimated using logistic regression analyses (Sabbioni et al. 2005). The OR without confounding factors of suffering from cataract were 6.4 times higher when the level of 4ADNT-Hb-adducts increased by one log-unit ($p<0.05$). Similar ORs were observed with hepatomegaly (7.6) and splenomegaly (9.6). From the z-values of the logistic regressions the probability $[p=1/(1+e^{-z})]$ of a negative health effect might be predicted for Hb-adduct levels found in exposed workers: (i) cataracts, $z=-2.90+1.85\times[\log(\text{Hb-4ADNT (ng/g)})]$; (ii) hepatomegaly: $z=-4.94+2.022\times[\log(\text{Hb-4ADNT (ng/g)})]$; (iii) splenomegaly, $z=-6.03+2.26\times[\log(\text{Hb-4ADNT (ng/g)})]$.

The distribution of health effects and biomarkers was then compared among the different worker groups (Table V). It appeared that the grinders had the highest frequency of cataract, splenomegaly, and hepatomegaly compared with the other groups. At the same time the highest adduct levels and urinary metabolite levels were found in the grinders. Therefore, the grinders seem to be at highest risk for developing TNT-related diseases.

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Table V. Comparison of health effects and biomarkers with job classification.

	Mixers	Loaders	Grinders	Packers	Controls
Cataract	64%	52%	70%	50%	26%
Splenomegaly	12%	8.3%	40%	0%	0%
Hepatomegaly	26%	16%	44%	0%	12%
ECG1	28%	24%	0%	0%	12%
ECG2	75%	78%	80%	100%	100%
ECG3	14%	14%	38%	17%	24%
Smoker	89%	93%	60%	67%	64%
Chromosomal aberrations[a]	2.5±1.6	2.6±2.2	3.3±1.0	3.7±1.5	3.2±0.7
Hb-4ADNT[b]	53 (13–184)	68 (11–883)	100 (47–249)	39 (7.0–69)	0 (0–45)
U-gl-4ADNT[c]	52 (10.4–243)	81 (36–497)	130 (41–289)	137 (2.2–207)	0.39 (0–2.8)
Air[d]	0.38 (0.29–1.5)	1.7 (0.75–4.0)	1.5 (0.98–3.6)	0.47 (0.38–0.56)	
Skin[e]	15 (6–33)	65 (17–1122)	82 (13–238)	40 (26–54)	

[a]Total, mean±SD.
[b]Median (range) (ng g^{-1}Hb).
[c]Median (range) (ng/100 µl).
[d]Median (range) (mg m^{-3}).
[e]Median (range) (mg).

Biological effects: chromosomal aberrations

The results of the chromosome aberration analyses of workers exposed to TNT and of the respective controls are presented in Table VI. There were no statistically significant differences in the various classes of chromosomal aberrations between the TNT-exposed subjects and the control group. The results suggest that TNT exposure in the ammunition factory had no clastogenic effects.

The main confounders for cytogenetic analysis are age and smoking habits. Therefore, the following statistical analyses were performed for the exposed worker group. The total frequency of chromosomal aberrations (chromosome-type+chromatid-type+gaps) was categorized in two groups (high and low) which were then compared for age and variables of smoking and exposure. Median values for age, the number of cigarettes smoked per day, and the number of smoking years were higher in the high cytogenetic damage group. In contrast the Hb-adduct levels were lower in workers with more cytogenetic damage. All comparisons were statistically not significant. There was a borderline significant correlation between age and cytogenetic damage (Spearman rank, $r=0.26$, $p=0.076$). In a further statistical analysis the workers were split into three age groups. The level of chromosomal aberrations in the youngest group was significantly lower than in the oldest group (Mann–Whitney *U*-test, $p<0.05$), although the mean Hb-adduct levels were higher in the young workers. In a next step the statistical analyses were performed with contingency tables. Age, work years and Hb-adducts were categorized in two groups. Smoking was categorized in smokers and non-smokers. The older worker group had an OR of 3 (0.98–9.5) compared with the younger workers as regards cytogenetic damage. Other ORs, for Hb-adducts, work years or smoker status did not yield significant values.

The genotypes of the exposed workers were compared with the cytogenetic damage using the Mann–Whitney *U*-test. For the *NAT1* genotype, a borderline significant relationship was found; the frequencies of chromatid breaks and total chromatid-type

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aberrations without gaps being higher in the *NAT1* rapid acetylator genotype (mean ± SD, 2.03 ± 1.42, and 2.10 ± 1.48, respectively; $n = 40$) than in the slow acetylator genotype (1.32 ± 2.01, $p = 0.053$ and 1.32 ± 2.01, $p = 0.05$, respectively; $n = 13$). When the results were compared by combining pairs of genotypes, the difference between the *NAT1* rapid and slow acetylators was only seen in *GSTT1* null ($p = 0.05$; $n = 3$) and *GSTM1* null workers ($p = 0.072$; $n = 5$) (data not shown). This suggests that *NAT1* rapid acetylators who have a homozygous defect in *GSTT1* or *GSTM1* genes show an increased sensitivity to chromosome damage associated with TNT exposure, which is in accordance with the observed increase in urinary mutagenicity among *NAT1* rapid acetylators. These results also appear to agree with our earlier study on workers exposed to various nitrotoluenes, where we saw an increase in chromosomal aberrations in *NAT1* rapid acetylators, but not in *NAT1* slow acetylators (Sabbioni et al. 2006). Similarly, the *GSTT1* genotype was observed to modulate the level of chromosome damage in the nitrotoluene-exposed workers. As the present findings are based on low numbers of subjects, further studies are needed to confirm if such genotype differences also exist in TNT-sensitivity.

Conclusions

The Chinese workers studied were exposed to high levels of TNT. The external dose correlated only moderately or not at all with the levels of any of the internal exposures as assessed by a variety of biomarkers. The urine of the exposed workers was mutagenic, indicating a systemic exposure to genotoxic agents, and the urinary mutagenicity correlated with Hb-adduct levels and urinary metabolite levels. In general, there was a weak correlation among urinary metabolites and Hb-adducts. The genotypes did not significantly influence the Hb-adduct levels. This might be the result of the substrate specificity of the different enzyme forms, which is generally unknown for the compounds and their metabolites examined in the present study. Furthermore, other studies have shown that at high exposure levels, genotype differences may play a minor role (Dallinga et al. 1998). However, TNT-exposed workers who carried the *NAT1* rapid acetylator genotype appeared to have increased urinary mutagenicity and (when combined with *GSTT1* or *GSTM1* null genotype) an elevated level of chromosomal aberrations. Health effects such as cataracts, splenomegaly and hepatomegaly, which are typical of TNT-exposed workers, related to the level of Hb-adducts. For cataracts, there was a relationship with the *GSTT1* genotype. Similar findings for *GSTM1* were earlier reported by Xu et al. (2002) who found that the overall GST activity of persons without the *GSTM1* gene was significantly lower in workers with cataracts than in control persons. Some clinical blood parameters related significantly with the Hb-adduct levels. The cytogenetic effects were affected by age but not by Hb-adduct levels.

The present results support the use of biomarkers such as Hb-adducts, urinary metabolites, and urinary mutagenicity to assess exposure and effect due to genotoxic exposure related to TNT. These biomarkers reflect exposure better than do external chemical measurements of TNT in air and on skin. The conclusions are supported by recent studies showing that selected metabolites of TNT are mutagenic (Grummt et al. 2006) and a meta-analysis showing that biomarkers are a more reliable measure of exposure than chemical measurements in air for occupational exposures (Lin et al. 2005).

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Table VI. Chromosomal aberrations per 100 cells in blood lymphocytes of workers exposed to TNT, factory controls, and laboratory controls.

		Mean (±SD) number of cells with chromosomal aberrations per 100 cells									
		Chromatid-type					Chromosome-type				
Group	Number of subjects	Gaps	Breaks	Exchanges	Total−gaps	Total+gaps	Breaks	Exchanges	Total	Total−gaps	Total+gaps
Exposed workers	54	0.44±0.63	1.81±1.60	0.07±0.26	1.89±1.63	2.34±1.64	0.17±0.38	0.18±0.43	0.35±0.56	2.23±1.72	2.71±1.25
Factory controls	7	0.43±0.53	1.86±1.34	0	1.86±1.34	2.28±1.60	0	0.43±0.79	0.43±0.79	2.40±0.96	2.71±1.79
Laboratory controls	26	0.35±0.63	1.66±1.74	0.13±0.49	1.79±1.81	2.13±2.03	0.27±0.53	0.08±0.27	0.35±0.63	2.13±1.94	2.48±2.13

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